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Title
ELECTRON MICROSCOPY OF RAT CEREBELLAR CORTEX FOLLOWING EXPOSURE TO IONIZING RADIATION

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The purpose of this paper is to describe the ultrastructural changes found in the irradiated cerebellar cortex of the rat following fixation by osmium perfusion. The cerebellum was selected for study because it contains a wide variety of easily recognized cellular types and structures in a small volume of tissue.

MATERIAL AND METHODS

A total of 25 adult Sprague-Dawley white rats was used. Before irradiation, the animals were anesthetized with sodium nembutal. The source of radiation was a constant potential X-ray therapy unit. The physical factors were: 250 kpv, 15 mA, 2 cm Cu filter. The entire head was exposed dorsally, with the body shielded. A single dose of 21.6 kR was given at 240 R/min. At 24 hours postexposure, the animals were anesthetized with sodium nembutal and the brain perfused by intravascular introduction of a chromic-osmium solution (Dalton, 1955). Use was made of a slight modification of the technique of Palay, et al. (1961), described elsewhere (de Estable, et al., 1964).

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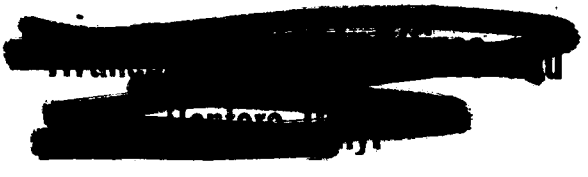
Following perfusion, the calvarium was opened and the brain removed. In successful perfusion the brain was homogeneously black. Slices from the cerebellum of these brains were embedded in paraffin for light-microscopy control study. Some sections were stained with PAS after pre-treatment with dimedone, as recommended by Bulmer (1959). Companion sections were subjected to enzymatic digestion with α-amylase. As a nuclear stain, hematoxylin or cresyl violet was used. Some brains were fixed in cold picroalcoholic fixative (Rossman) and others in Zenker-formol.

From the chromic-osmium fixed animals, pieces of cerebellum adjacent to those used for light-microscopy control were placed in the same cold fixative and kept in the ice box for 2 hours. The tissue was then embedded in Epon 812 according to the method of Luft (1961). Thin sections were made with glass knives applied to the Porter-Blum "M1" ultramicrotome. Sections mounted on carbon-coated formvar grids were then stained with lead hydroxyde according to the method of Karnovsky (1961) or by a combined procedure utilizing saturated aqueous uranyl acetate for 1 hour and then lead hydroxyde for 15 minutes. Thick sections were made and mounted on glass slides and stained with paraphenylenediamine (Estable-Puig, et al., 1963) and examined with the light microscope. Selected sections were stained with PAS according to the procedure of Munger (1961). Some were observed with the phase microscope. Thin sections were examined with an HU-11 Hitachi electron microscope at a voltage of 50 or 75 kv.



RESULTS

Light Microscopy Study of Irradiated Cerebellar Cortex.- The myelin core of the cerebellar folia, including myelinated fibers, astrocytes, and interfascicular oligodendrocytes, appeared free from change (Figs. 1, 2). In the granular layer, numerous altered granule cells were noted. They were randomly distributed; as a rule they were isolated but now and then were present in groups. The nucleus of these cells was greatly reduced in size and intensely hyperchromatic (Figs. 3, 4). PAS-positive material was found occasionally in the more superficial part of the granular layer, between granule cells. No changes were found in Golgi cells or in glia except for slight enlargement of some astrocytes.

Figs. 1, 2

Figs. 3, 4

Purkinje cells appeared normal. In sections of cerebellum fixed by immersion, some Purkinje cells were dark and others showed cytoplasmic chromatolysis and vacuolation, but since these changes were also noted in sections from control animals they were interpreted as artifacts due to the fixation. They were not seen in the perfusion-fixed cerebellum (Figs. 5, 6). The nuclei of the Bergmann cells appeared normal. The perikaryon was crowded with PAS-positive material corresponding histochemically to glycogen, as proved in amylase and dimedone control sections. The glycogen was also homogeneously distributed in the osmium-fixed specimens (Figs. 7, 8).

Figs. 5, 6

Figs. 7, 8

In the molecular layer few changes were seen. Some nuclei were poor in chromatin and in some brains the basket cells looked like empty shells. The nuclei of the glial cells appeared unaltered, but such cells, presumably astrocytes, were sometimes surrounded by small amounts of glycogen.

Occasional pyknotic nuclei were seen. In the deeper two-thirds of the molecular layer, large amounts of PAS-positive material were present in the form of parallel rows extending to the superficial part of the molecular layer. As based on amylase and dimedone control studies, this PAS-positive material was interpreted as glycogen. In tissue fixed by osmic perfusion, the glycogen was homogeneously distributed in the cells, while in picroalcoholic-fixed tissue it had a coarse granular appearance. This has been previously demonstrated (de Estable, et al., 1964).

Cerebellar vessels were dilated due to the fixation by perfusion.

No morphological changes were observed in the meninges. A few arachnoid cells contained some PAS-positive material.

Electron Microscopy Study of Irradiated Cerebellar Cortex.- The myelin core of the cerebellar folia appeared normal. The myelinated fibers were well preserved, as in Fig. 9. Mossy fibers were also unaltered. Vessels in such regions appeared free from change. Fig. 9

In the granular layer, altered granule cells were randomly distributed among intact granule cells. In few granule cells were intermediate stages between slight involvement to frank necrosis observed. In slightly involved cells chromatin clumping in the nucleus was the only change observed (Fig. 10). In severely affected cells most of the nuclear chromatin was condensed into a homogeneous mass, and the size of cells thus affected was greatly reduced, sometimes by as much as 50 percent. Nucleoli in such cells were not recognizable. Lacunar areas containing granular material occupied as much as one-fourth the nuclear cross section. Vacuole-like structures were also visible and frequently were close to the nuclear membrane. Now and then the nuclear membrane was serrated and focally disrupted. The cytoplasm was shrunken and more dense than usual and Fig. 10

ribosomes were clumped. This was noticed mainly at the site of origin of dendrites (Fig. 10). In close relationship with some granule cells were processes of astrocytes, which were enlarged and loaded with deeply staining granules which corresponded to glycogen (Fig. 11). In some sections the outer surface of severely damaged cells seemed to have a multilayered border, probably representing membrane injury, as seen in Fig. 12. In this layer perivascular astrocytic processes loaded with glycogen were seen (Fig. 13). As far as could be determined, no structural changes were seen in axons of the granule cells, including the parallel fibers in the molecular layer. (Figs. 11-13)

Purkinje cells retained their usual structure. The nucleus was roughly circular and its membranes showed some indentations and foldings. A double-layered nuclear membrane is clearly visible as are also nuclear pores in tangentially sectioned cells (Fig. 14). The nuclear material was granular in some cells, but chromatin clumping was evident in others (Figs. 15, 15A). A large nucleolus with a juxtanucleolar body is to be seen in Fig. 16. In Fig. 17 parallel membranes are to be seen in the granular endoplasmic reticulum as well as ribosomes. The mitochondria are well preserved and their cristae, as usual, run parallel with the long mitochondrial axis. Some areas at the periphery of the cytoplasm contain cisternae in association with mitochondria, as has been noted in the normal cerebellum by Herndon (1963). The Golgi apparatus and the lysosomes are also preserved. Surrounding the Purkinje cells, various structures can be seen in close apposition with the cytoplasmic membrane. These include synaptic contacts (with basket fibers), some of normal appearance and others showing synaptic-vesicle clumping. (Figs. 14-17)

Close to the Purkinje cells, Bergmann cells were observed (Fig. 14). The nuclei were round and the chromatin was regularly distributed. The perikaryon was enlarged and loaded with glycogen granules, distributed homogeneously between the cytoplasmic subcellular organelles (Fig. 18). Present at the same level were basket-cell fibers which were free from glycogen deposits. Fig. 18

In the molecular layer no pathological changes were detected except for enlargement of some astrocytes and except for the presence of myriad glycogen granules in the Bergmann-cell processes (Figs. 19-21). Figs. 19-21

No pathological changes were found in the meninges.

DISCUSSION

Incidence and degree of damage incurred by various components of cerebellum 24 hours after exposure to 21.6 kR X radiation varied widely. The granule cell was the most radiovulnerable cerebellar element. As has been recognized previously in the rat, clumping of nuclear chromation occurred in earlier stages of injury, while in later stages the chromatin was reduced to a homogeneous mass, indicating frank nuclear necrosis. The clear lacunar areas in the nucleus of cells thus damaged corresponded to the light microscopy image of karyorrhexis. Membrane duplication and blebbing noted in the present study were also seen by Pitcock (1962) in the rat as soon as 5 minutes after exposure to 15 kR high-intensity γ radiation. Some of our observations confirmed those by Vogel (1959, 1962) in the rabbit following exposure to γ radiation at 15 kR, namely that at the 24-hour stage, intranuclear granules were clumped and nuclear membranes serrated. We could not, however, find the broadening of the cytoplasmic space he described. Of particular interest

in our material was the lack of change in the axonal processes of the damaged granule cells at this stage.

Purkinje cells suffered much less alteration than granule cells, and the incidence of their involvement was also lower. The chief alteration was that of clumping of nuclear chromatin into granular aggregates, which was considered pathological. There were also some indentations and foldings in the nuclear membrane, but whether these represent pathological changes is not evident. Moreover, synaptic vesicles around the Purkinje cell body were sometimes clumped, suggesting an abnormality in synaptic contact.

Low radiosensitivity of Purkinje cells as compared with granule cells is well recognized, for example, from the observation by Hager, et al. (1962) that Purkinje cells in the hamster were free from change at 22 hours following exposure to 40.2 kR X radiation, at a time when granule cells were shrunken and showed clumping of nucleoplasm and at a time when capillaries exhibited endothelial-cell swelling, outpouchings, and other irregularities.

The radiosensitivity of granule cells is comparable with that of granular cells of the olfactory bulb and with that of lymphocytes. The greater sensitivity of granule cells over Purkinje cells appears to be related to their respective innate qualities and differing restitutive processes. In all tissues nuclear histone and globulin content are reduced following irradiation, and it has been suggested that bonds between histone and DNA are broken by radiation, but in less degree for nerve cells than for other cells, and that this might be a basis on which nerve cells are the more radioresistant (Ernst, 1962). A study of

H^3-leucine incorporation in cerebellar cells a few hours following exposure to proton radiation has revealed a significantly decreased uptake of this precursor by granule cells and a fourfold uptake by Purkinje cells (Zeman and Curtis, 1962), signifying underlying differences in synthetic processes in the two cell types.

Of perhaps greatest interest in our preparations were the changes observed in Bergmann cells. The cytoplasm of these cells was considerably enlarged, and the enlargement appeared to be due solely to the accumulation of glycogen granules. Otherwise the cytoplasm appeared unaltered, and no changes were seen in the nucleus but a blurred image of the nuclear membrane. The increase in size of the cytoplasm corresponded to that noted in the hamster by Hager, et al. (1962) at 15 hours after exposure to 15 kR X-rays.

Glycogen granules have been observed by light microscopy in the cerebellar cortex of the rat about 12 hours following 48-Mev a-particle irradiation at a peak dose of 30 kR given at 1 kR/min (Klatzo, et al., 1961), and in the brain of the rat (cerebellum not specifically mentioned) following exposure to as small an X-ray dose (250 kvp) as 1.2 kR given at 115 R/min (Miquel, et al., 1963). Dose-rate effective limits of 3 kR X radiation (1000 kvp) have also been established: glycogen granules appeared at 0.6 kR/min but not at 0.3 kR/min (Brownson, et al., 1963). Glycogen accumulation is regarded as a pathological change, the increase being the outcome, apparently, of an inability of astroglia to utilize or transfer the glucose coming to them from the blood stream. What bearing such accumulation might have on Purkinje and other nerve cells is not known.

Vessels in the cerebellum in our series of rats showed no visible change at the time period at which observations were made, namely at 24 hours, and thus glycogen accumulation appears to have occurred in the absence at least of morphological changes in vessels. Under conditions of 15 kR γ radiation, in the rat, Pitcock (1962) indicated that endothelial swelling was evident at 24 to 48 hours postexposure. According to his descriptions and figures, this endothelial swelling is linked with swelling of the "process of the clear glial cells adjacent to blood vessels." These changes might represent fixation artifacts.

No other electron microscopy data on relative radiovulnerability of cerebellar vessels up to the 24-hour stage seem available.

Other elements in the cerebellum in our series appeared unaltered, including the various cells in the molecular layer. White matter and its oligodendroglia also appeared unaltered, and it is of interest that myelin in peripheral nerves of the rat as observed electron microscopically is also relatively radioresistent (Andres, 1963). No inflammatory-cell reaction occurred, as is characteristic for the rat even when exposed to a massive dose of radiation (Hicks, Wright and Leigh, 1956). Taking all these points into consideration, the cellular effects observed were considered to be due to ionizing events in the cells, not to alterations in the milieu incident to primary vascular injury.

SUMMARY AND CONCLUSIONS

The observations dealt with in this report were on the cerebellum of rats exposed 24 hours previously to 21.6 kR X radiation given in a single dose. At this time period, as noted by electron microscopy,

scattered granule cells were severely damaged, the nuclei of some Purkinje cells showed minor changes, and the cytoplasm of Bergmann glia was packed with glycogen granules. Other elements were little if at all affected.

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FIGURE LEGENDS

Fig. 1. Fixation by perfusion with chromic-osmium solution. Epon embedding. Thick section stained with paraphenylenediamine and observed under phase contrast. Area of the myelin core of a cerebellar folium, showing well preserved and closely packed myelinated fibers and a fibrous astrocyte of normal appearance. X 1665.

Fig. 2. Area similar to that illustrated in Fig. 1, showing an intact oligodendrocyte in relation to normal myelinated fibers. Thick section stained with paraphenylenediamine. X 1500.

Fig. 3. Fixation by perfusion with chromic-osmium solution. Epon embedding. Thick section stained with paraphenylenediamine and observed under phase contrast. Area of the granular layer, showing scattered pyknotic granule cells. Notice also the empty dilated vessel and the normal appearing myelinated fibers and a glomerulus. X 1665.

Fig. 4. Area similar to that illustrated in Fig. 3. Four pyknotic granule cells are to be seen. Notice also the two cerebellar glomeruli, which appear normal. Paraphenylenediamine stain. X 1500.

Fig. 5. Fixation by perfusion with chromic-osmium solution. Epon embedding. Thick section stained with paraphenylenediamine stain. The two Purkinje cells appear normal. X 1500.

Fig. 6. Fixation by perfusion with chromic-osmium solution. Epon embedding. Thick section stained with paraphenylenediamine. The Purkinje cell is of normal appearance. Granule cells are damaged. X 1500.

Fig. 7. Fixation by perfusion with chromic-osmium solution. Paraffin embedded section stained with PAS-hematoxylin after pretreatment with dimedone. Notice the abundant PAS-positive material corresponding to perikaryon and cytoplasmic processes of Bergmann cells. X 580.

Fig. 8. Higher magnification of a field illustrated in Fig. 7. Notice that the glycogen is restricted to the cytoplasm of Bergmann cells and is not present in the cytoplasm of Purkinje cells. Also numerous pyknotic granule cells can be seen. X 720.

Fig. 9. Fixation by perfusion with chromic-osmium solution. Epon embedding. Boundary region between myelin core and granular layer. Note the unaltered myelinated fibers, which contain mitochondria (M) and neurofilaments. Also visible is a mossy fiber (MoF), which has synaptic contacts with granule-cell dendrites (S). Notice also the presence of a glial fiber (GF) close to a granule cell (G). Lead staining according to Karnovsky. X 13000.

Fig. 10. Fixation by perfusion with chromic-osmium solution. Epon embedding. Some granule cells (G) present a normal structure, except possibly for some clumping of nuclear material. Two granule cells are greatly reduced in size and their nuclei have an increased density and contain lacunar areas. Disruptions and "blebbing" of the nuclear membrane have occurred. Small vacuole-like areas are to be seen near the nuclear border. The cytoplasm is shrunken and is denser than normal and ribosomes are clumped. A cerebellar glomerulus (CG) of normal appearance is also visible. X 10000.

Fig. 11. Higher magnification of a granule cell (G) in the same general region as illustrated in Fig. 10. Some astrocytic processes (A) are loaded with glycogen granules (GI). Vacuole-like structures are present along the nuclear membrane and in the interior of the nucleus. X 35000.

Fig. 12. From some general region as in Fig. 11. To the right is an altered granule cell (G). The arrows point to a multilayered cytoplasmic membrane. X 25000.

Fig. 13. Fixation by perfusion with chromic-osmium solution. Epon embedding. Area of molecular layer. An empty vessel is shown, and close to it is an astrocytic process (A) loaded with glycogen granules (GL). Arrows point to an axo-dendritic synaptic contact (S). Lead staining according to Karnovsky. X 35000.

Fig. 14. Fixation by perfusion with chromic-osmium solution. Epon embedding. Area of Purkinje-cell layer. In the upper left is a Purkinje cell (P) with an indented nucleus. Nuclear pores (Po) (arrows) are visible. Cytoplasm is well preserved and contains mitochondria (M), Golgi apparatus (GA), granular endoplasmic reticulum (ER), and lysosomes (L). At the lower right is a Bergmann cell (B) with a round nucleus and perikaryon and two cytoplasmic processes laden with glycogen granules (GL). Other glycogen-granule-laden glial processes are also to be seen. Close to the cell are myelinated fibers and basket fibers (BF). At the lower left is part of another Purkinje cell. Uranyl acetate and lead stain. X 9000.

Figs. 15 and 15A. Area similar to that illustrated in Fig. 14. Higher magnification of a Purkinje cell (P). The arrows indicate sites of synaptic contact between basket fibers and the perikaryon of a Purkinje cell. The Golgi apparatus (GA) and endoplasmic reticulum (ER) appear normal. In Fig. 15A, part of a nucleus (N) is shown. Notice the chromatin clumping, which is considered abnormal. Uranyl acetate and lead stain. X 12000 and 30000, respectively.

Fig. 16. Area similar to that in Fig. 15. Higher magnification of a Purkinje cell. The nuclear material is unevenly distributed, suggesting abnormality. A normal appearing nucleolus (NC) and paranucleolar body are visible. Uranyl acetate and lead stain. X 20000.

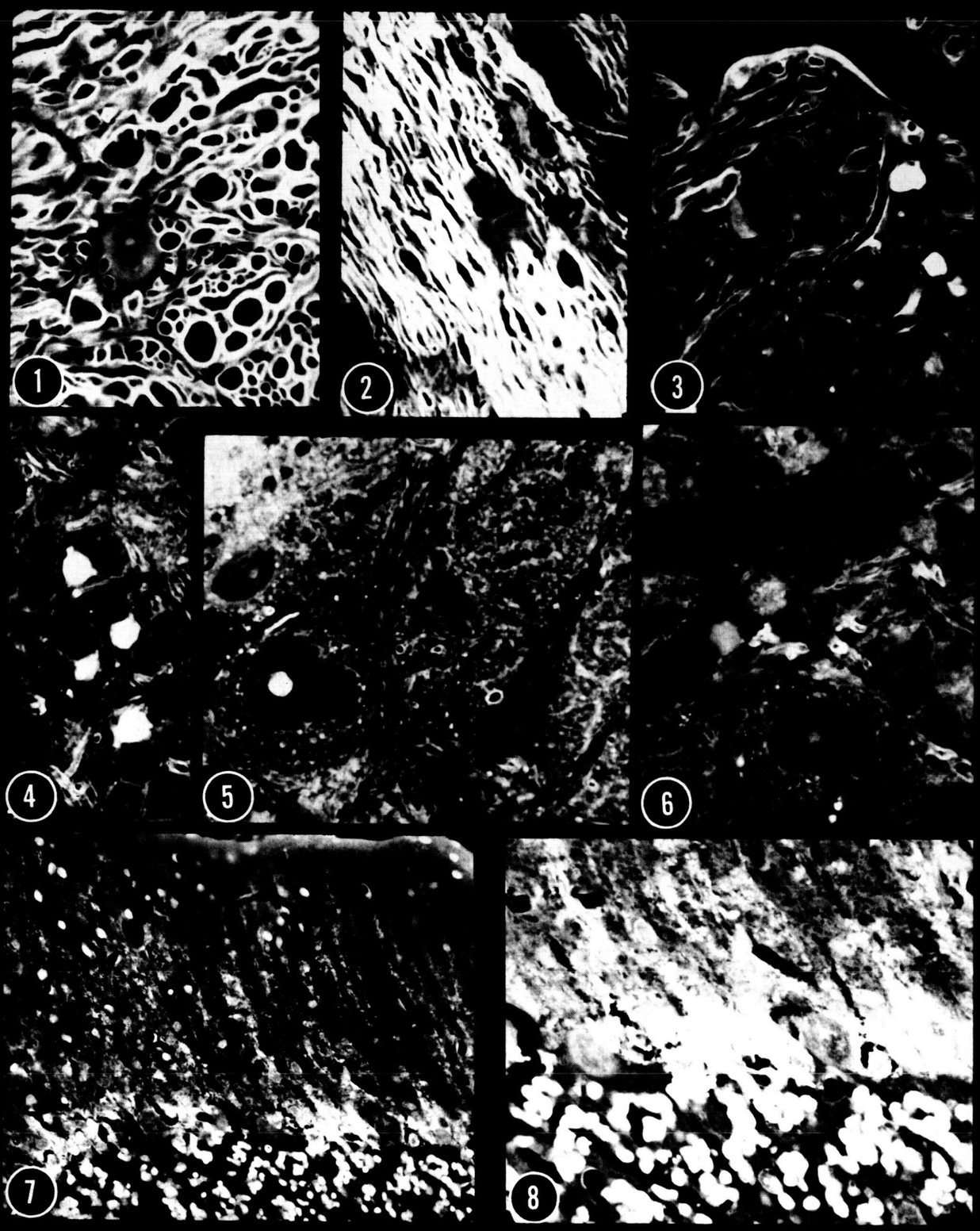
Fig. 17. Similar preparation as in Fig. 16. Purkinje cell (P). The arrows point to synaptic contacts (S) between basket fibers (BF) and a Purkinje cell. Notice in the axonal terminal the clumping of synaptic vesicles. The Purkinje-cell nucleus (N) appears normal. Uranyl acetate and lead stain. X 20000.

Fig. 18. Fixation by perfusion with chromic-osmium solution. Region of Purkinje and Bergmann cell layer. Two Bergmann cells (B) are to be seen. Their cytoplasm is loaded with glycogen granules (GL), which are diffusely distributed. Basket fibers (BF) are free from glycogen granules. Part of a Purkinje cell (P) is visible at the lower right. Epon embedding. Uranyl acetate and lead stain. X 12000.

Fig. 19. Fixation by perfusion with chromic-osmium solution. Epon embedding. Molecular layer. An astrocytic process (A) is enlarged and loaded with glycogen granules (GL). Uranyl acetate and lead stain. X 20000.

Fig. 20. Fixation by perfusion with chromic-osmium solution. Epon embedding. Adjacent to a dendritic branch of a Purkinje cell is an astrocytic process (A) loaded with glycogen (GL). To the right are parallel fibers (PF), and above are Purkinje-cell dendrites (PD). Uranyl acetate and lead stain. X 20000.

Fig. 21. Fixation by perfusion with chromic-osmium solution. Epon embedding. Molecular layer. Glycogen (GL) and glial fibers (GF) are present in the same astrocytic process (A). At the bottom are several axo-dendritic synaptic contacts (S). Lead staining according to Karnovsky. X 30000.



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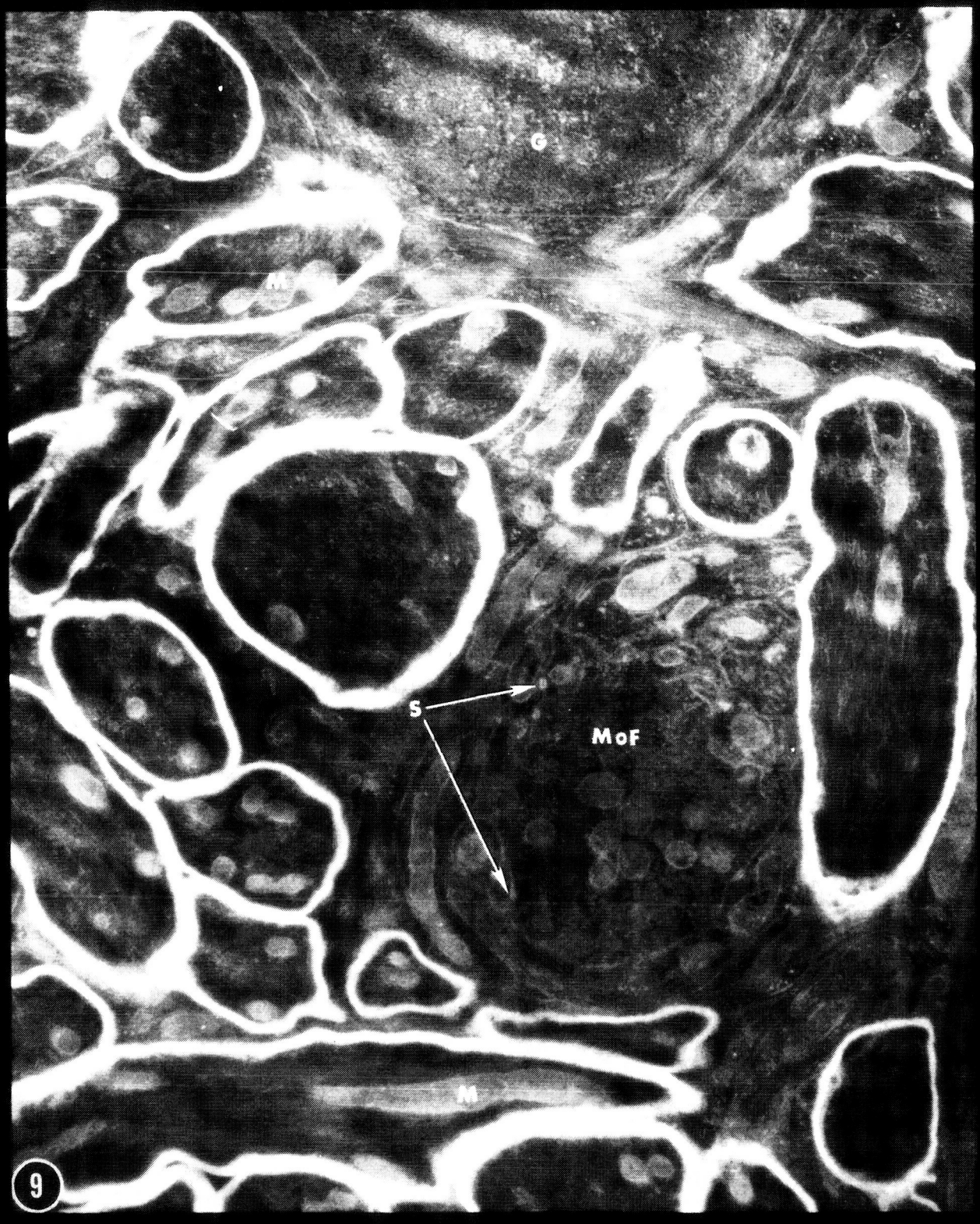

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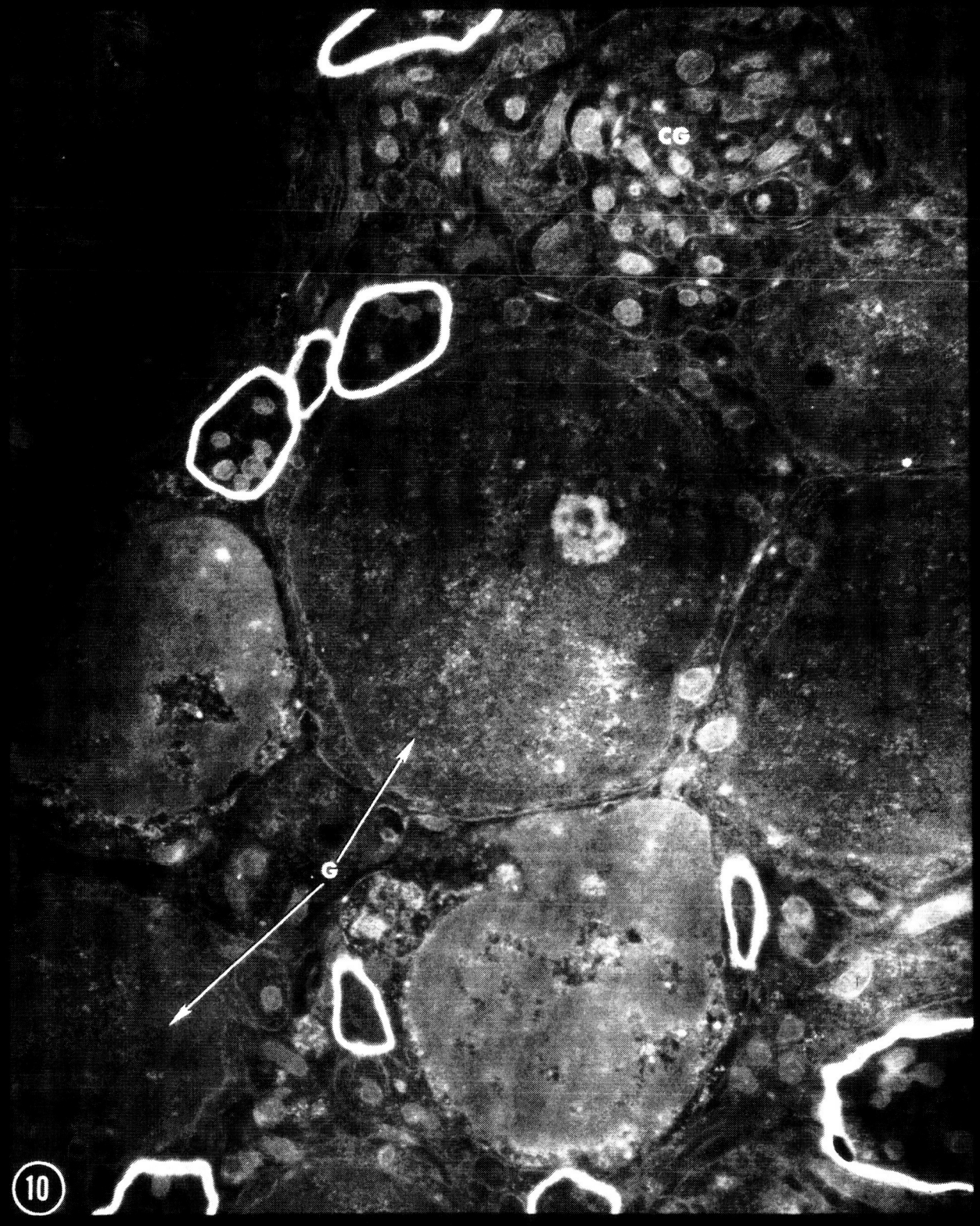

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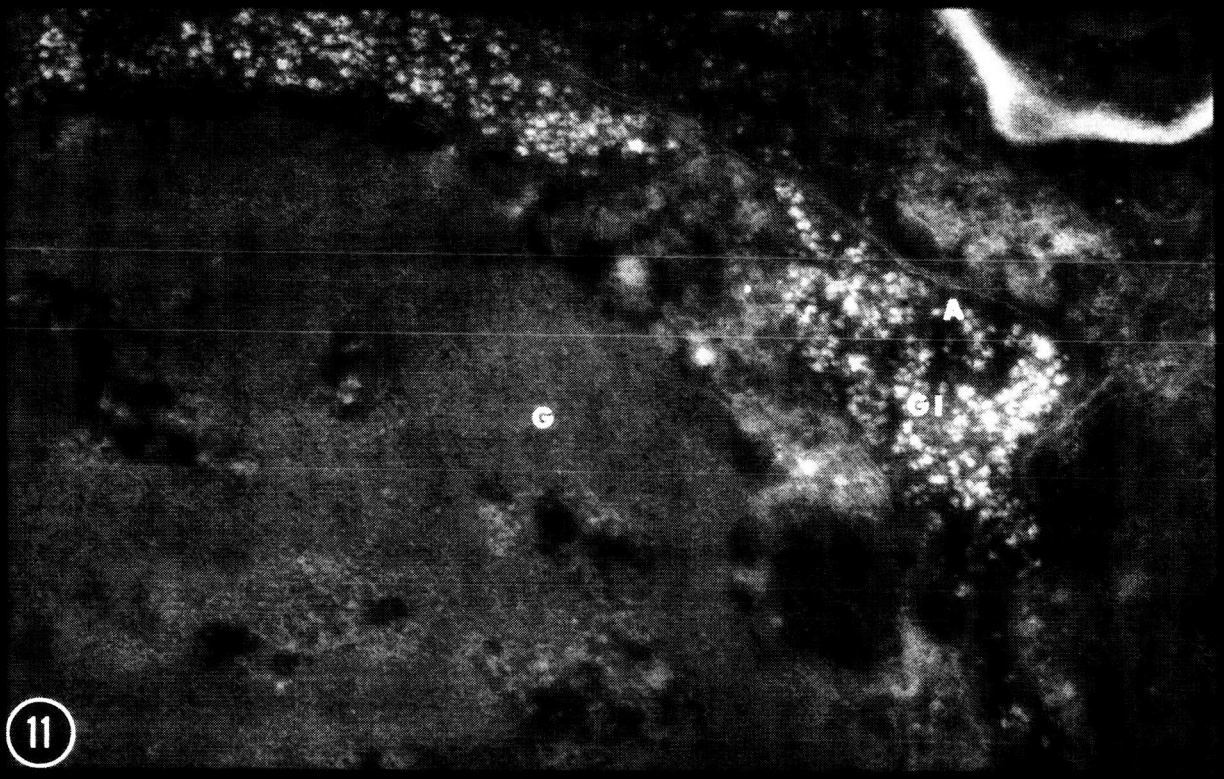

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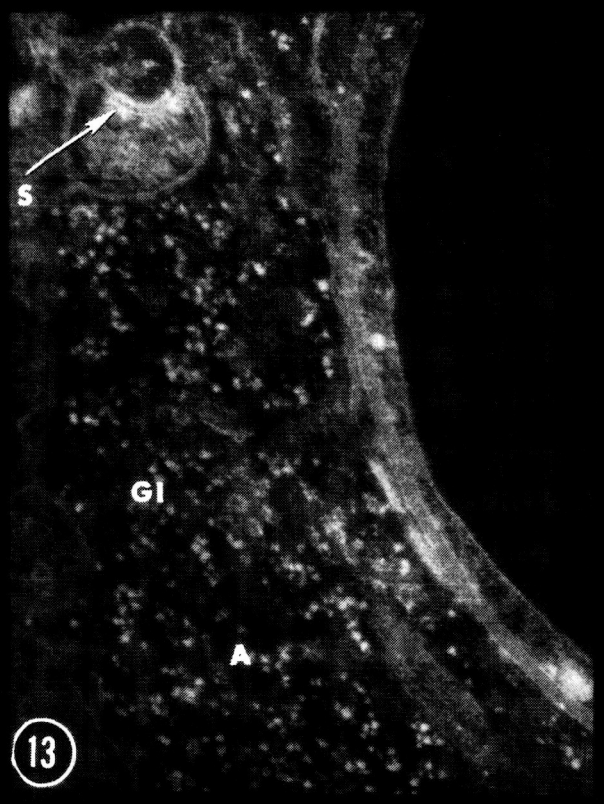

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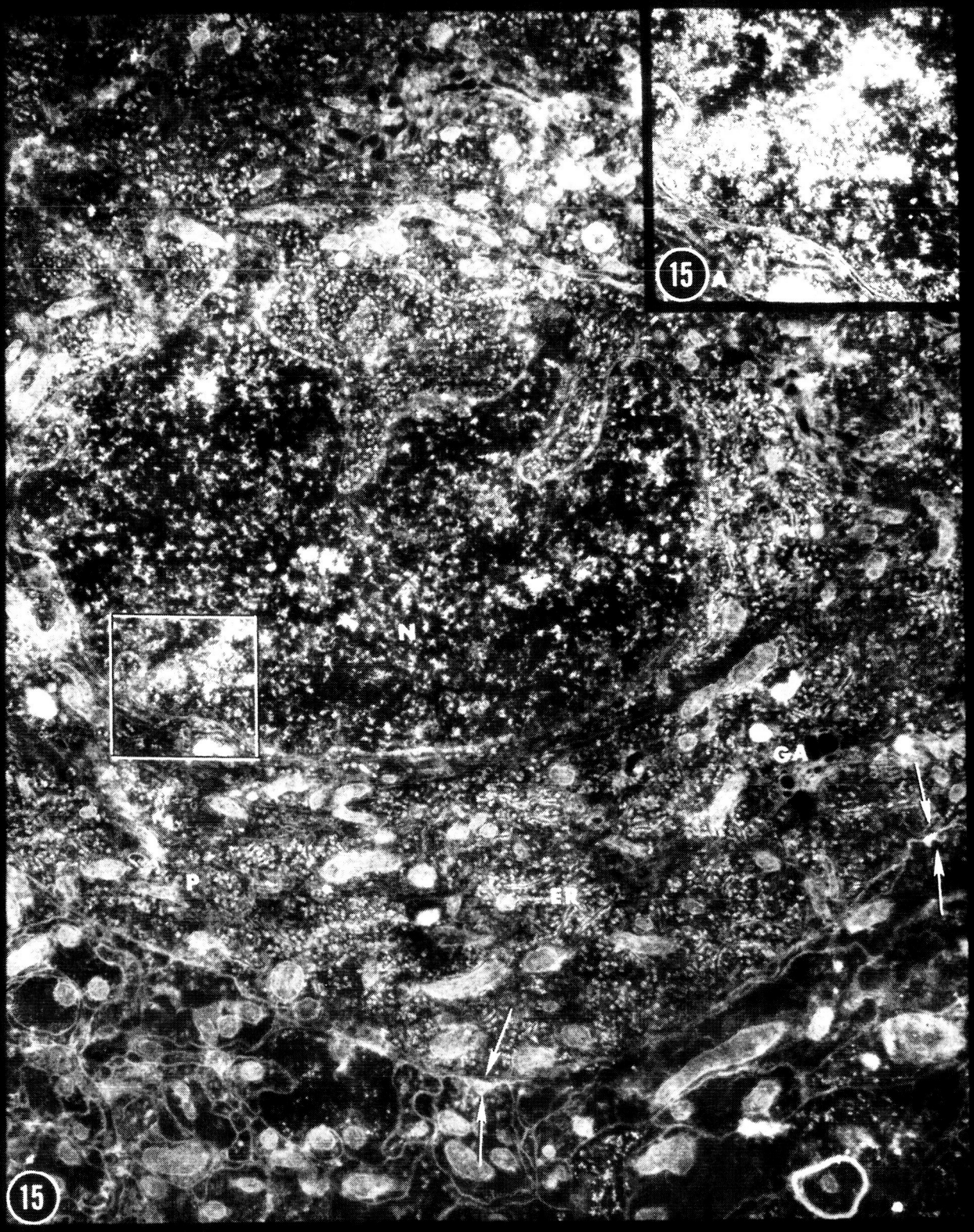

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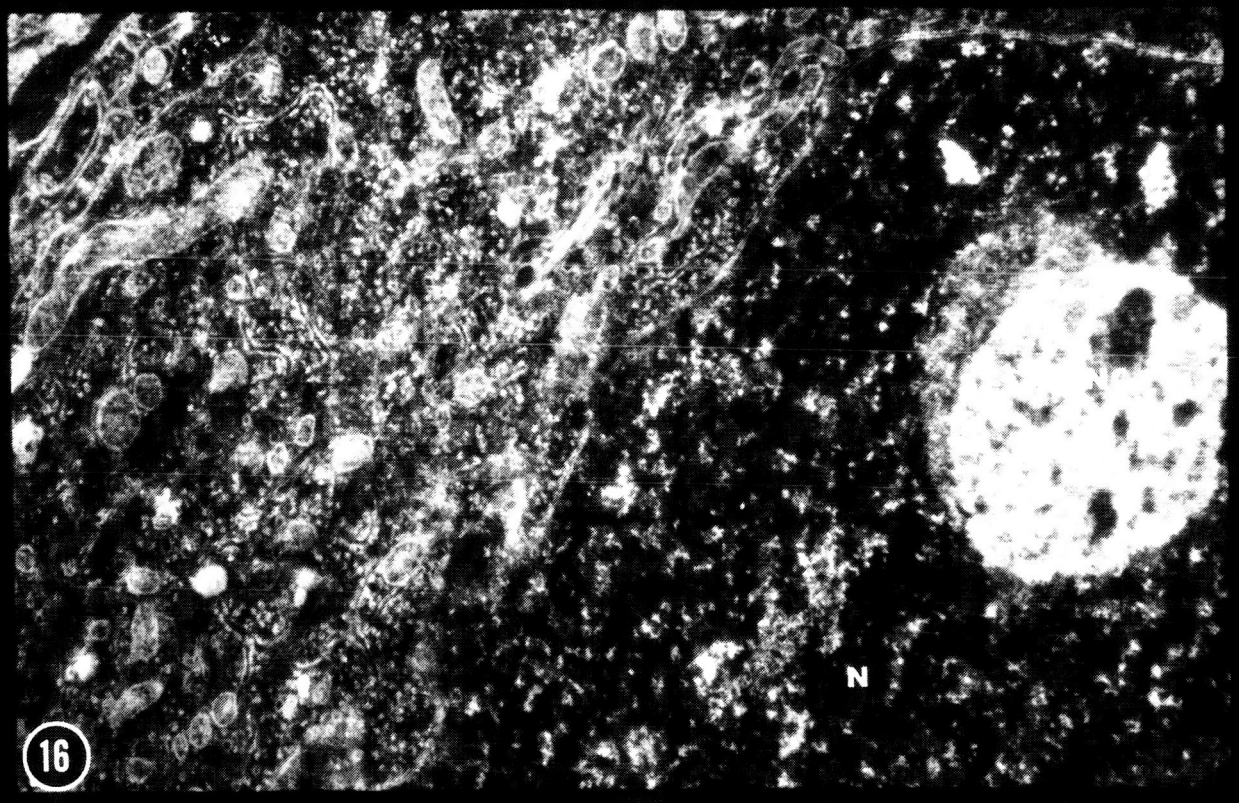

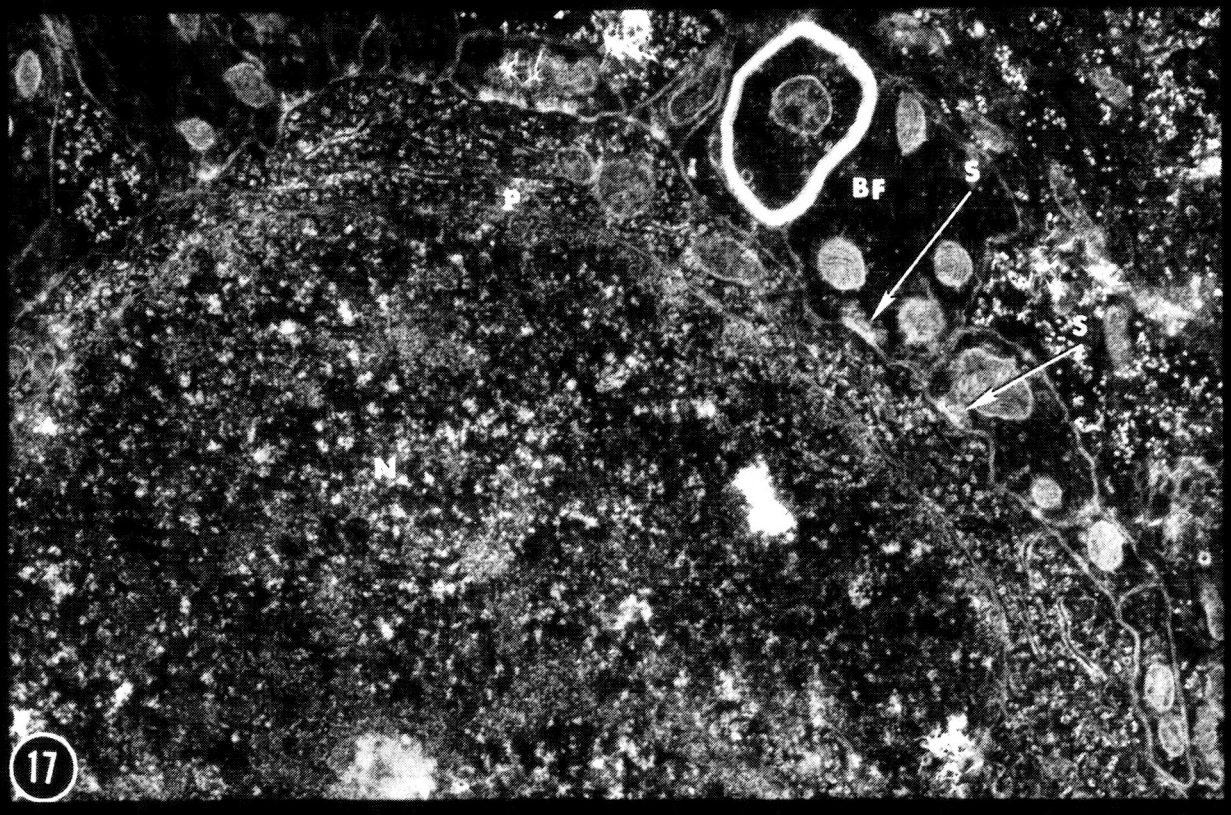

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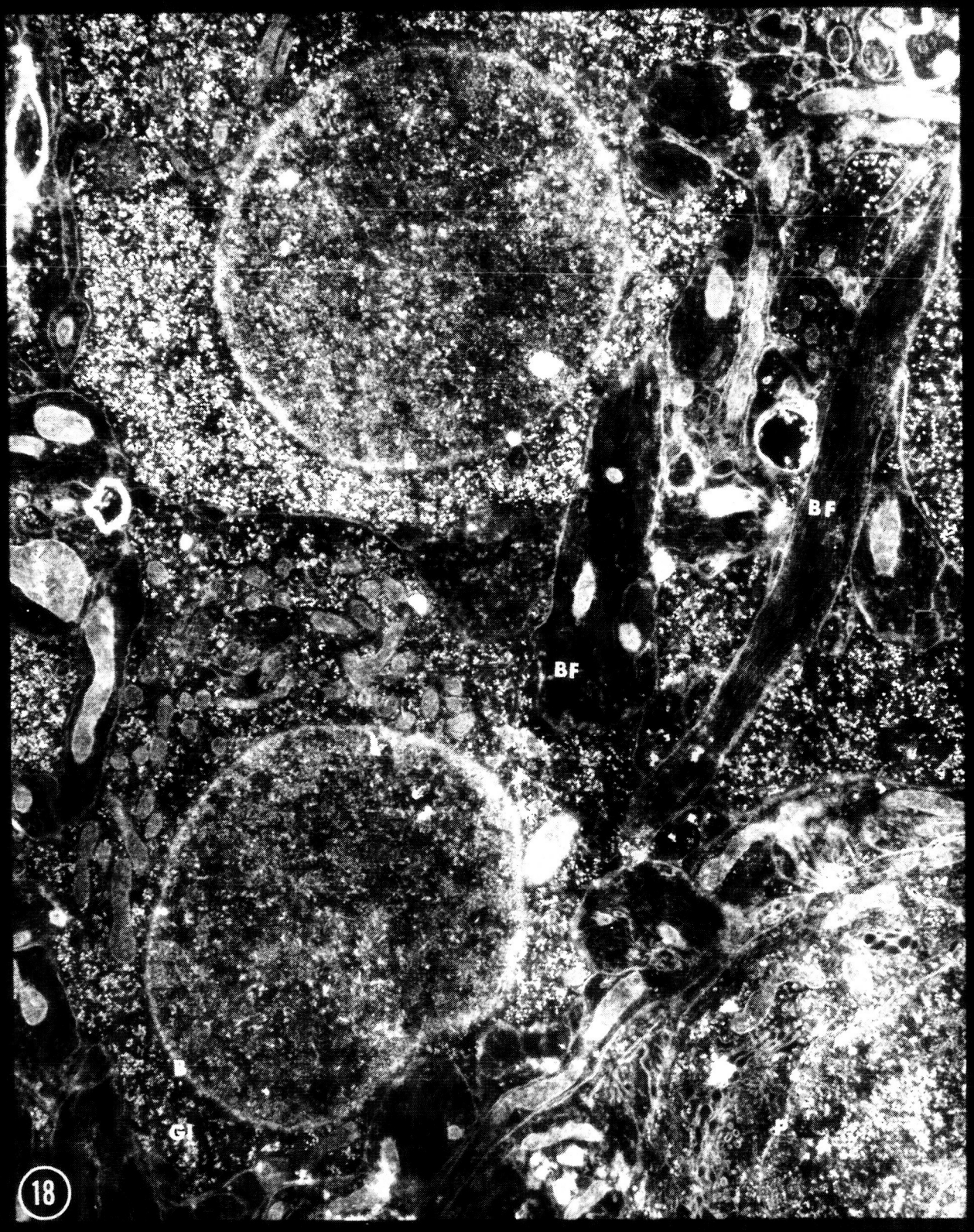

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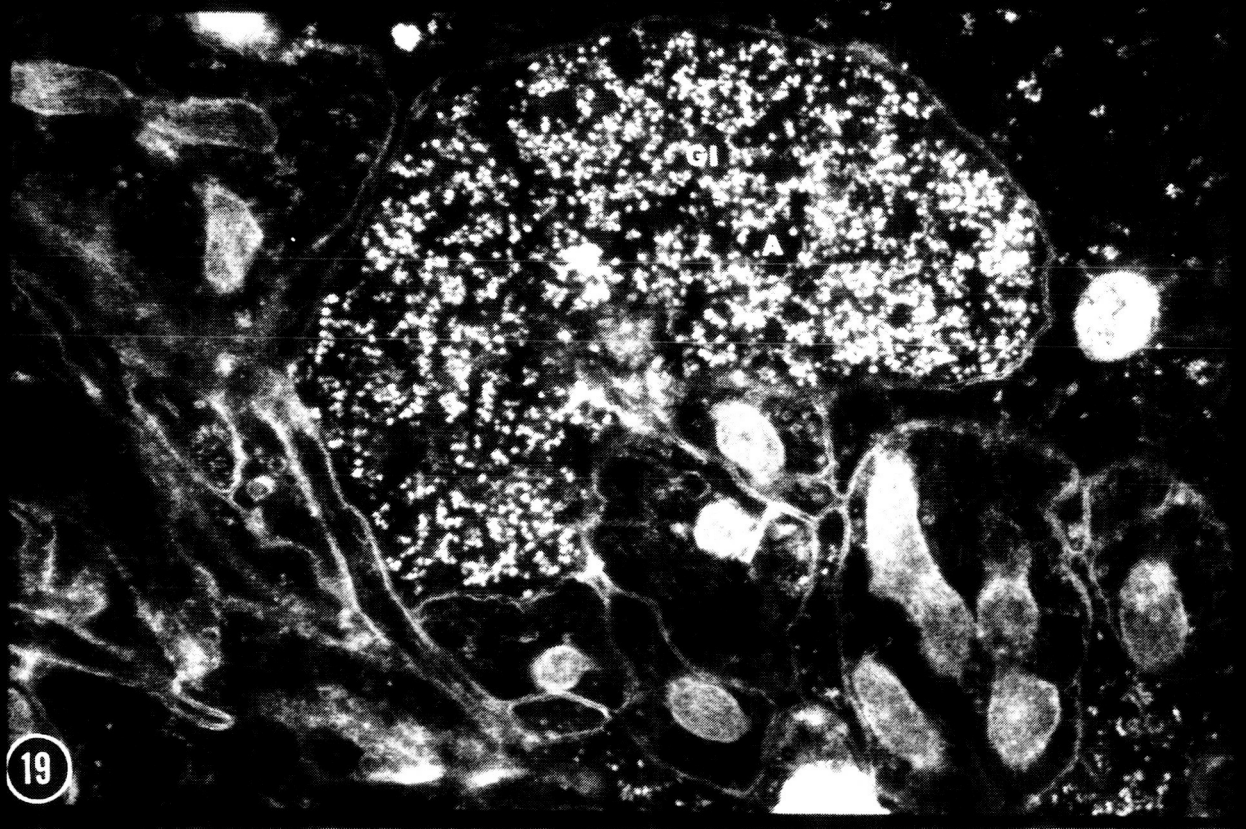

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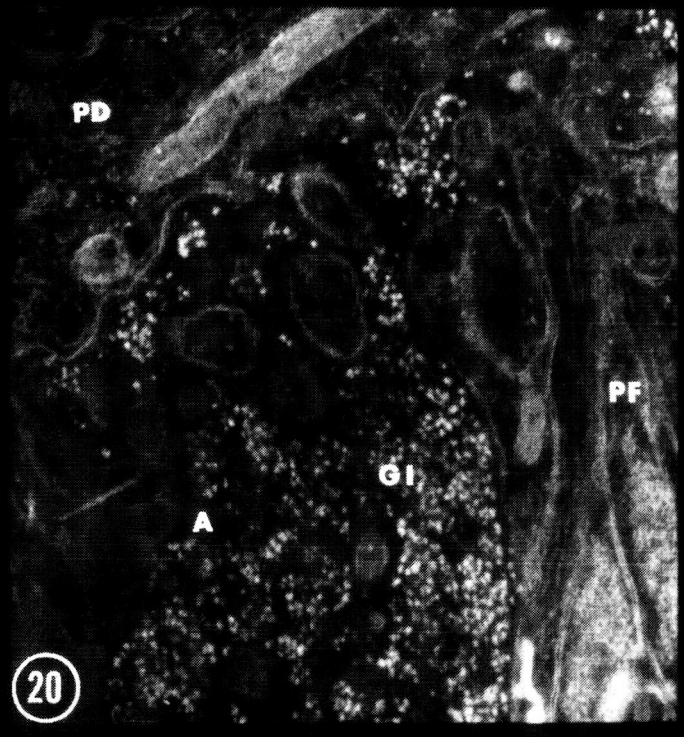

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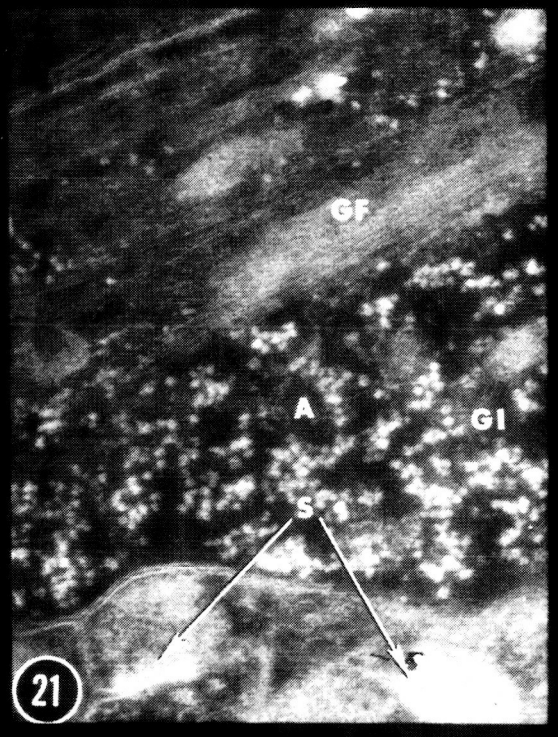

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